

AD_____

AWARD NUMBER: W81XWH-05-1-0417

TITLE: Does Combination Immunotherapy with Human Monoclonal Antibodies Against HER2 and CXCR4 Augment Breast Cancer Killing In Vitro and In Vivo?

PRINCIPAL INVESTIGATOR: Wayne A. Marasco, M.D., Ph.D.

CONTRACTING ORGANIZATION: Dana-Farber Cancer Institute
Boston, Massachusetts 02115

REPORT DATE: August 2006

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				<i>Form Approved</i> OMB No. 0704-0188	
<small>Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.</small>					
1. REPORT DATE (DD-MM-YYYY) 01-08-2006		2. REPORT TYPE Annual		3. DATES COVERED (From - To) 15 Jul 2005 – 14 Jul 2006	
4. TITLE AND SUBTITLE Does Combination Immunotherapy with Human Monoclonal Antibodies Against HER2 and CXCR4 Augment Breast Cancer Killing In Vitro and In Vivo?				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-05-1-0417	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Wayne A. Marasco, M.D., Ph.D. E-Mail: wayne_marasco@dfci.harvard.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Dana-Farber Cancer Institute Boston, Massachusetts 02115				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT The chemokine receptor CXCR4 and its ligand CXCL12 (SDF1α) have been proposed to regulate the directional migration and invasion of breast cancer cells to sites of metastasizes. The CXCR4 molecule could be a potential target to control breast cancer. Human epidermal growth factor receptor-2 (HER2) overexpression contributes to tumor progression and metastasis. A humanized monoclonal antibody Herceptin (Trastuzumab) is currently in clinical use. Thus, both of CXCR4 and HER2 play important roles in breast cancer progress, the linkage between CXCR4 and HER2 has also been reported. HER2 upregulates the expression of CXCR4, which is required for HER2-mediated lung invasion and metastasis. Therefore, we aimed to assess the anti-tumor effects of combinational immunotherapy by targeting both CXCR4 and HER2 in vitro and in a nude mice breast cancer model. The result from this study should provide pre-clinical data that may ultimately aid in testing the hypothesis that additive or synergistic effects of combinational treatment with anti-CXCR4 and anti-HER2 human Mabs may lead to an additive or synergistic effect in human clinical trials of breast cancer. We have produced enough antibodies for the entire study, and established the necessary cell lines for both in vitro and in vivo studies. We have evaluated the effects of CXCR4 Mabs in combination of Herceptin or alone on inhibition of chemotaxis, invasion and proliferation on breast cancer cells. The results and experience we have obtained through these studies will lead us to answer the question we have proposed and guide us to perform the in vivo studies which will be started in the next year.					
15. SUBJECT TERMS antibody, immunotherapy, Her2, CXCR4					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			USAMRMC
			UU	8	19b. TELEPHONE NUMBER (include area code)

Table of Contents

Cover.....	1
SF 298.....	2
Introduction.....	4
Body.....	4-6
Key Research Accomplishments.....	7
Reportable Outcomes.....	7
Conclusions.....	7
References.....	7
Appendices.....	7

Introduction:

The chemokine receptor CXCR4 and its ligand CXCL12 (SDF1 α) have been proposed to regulate the directional migration and invasion of breast cancer cells to sites of metastases (1). Inhibiting the interactions of CXCL12/CXCR4 either by antibodies against CXCR4 or small molecule antagonists impairs breast cancer metastasis in a mouse model. In addition to its role in breast cancer metastasis, an essential role of CXCR4 in breast cancer growth has been proposed by several studies. The CXCL12-CXCR4 signaling pathway is required for the regulation of the growth and the survival of both primary breast cancer cells and invasive or micrometastatic tumor cells. Inhibiting CXCR4 with RNAi, or the specific antagonist, substantially delayed the growth of breast cancer cells in SCID mice (2,3). Therefore the CXCR4 molecule could be a potential target to control breast tumor metastasis as well as growth.

Human epidermal growth factor receptor-2 (HER2), which is overexpressed in about 30% of all breast cancers, has been a target for antibody-based therapy for advanced breast cancer. A humanized monoclonal antibody Herceptin (Trastuzumab) is currently in clinical use. Despite careful patient selection on the basis of ErbB2 expression, only a minority of patients respond to trastuzumab monotherapy (4).

A study recently showed that HER2 upregulates the expression of CXCR4 by inhibiting CXCR4 degradation, which is required for HER2-mediated lung invasion and metastasis. A significant correlation between HER2 and CXCR4 expression was observed in human breast tumor tissues. Similar to HER2, CXCR4 expression correlated with a poor overall survival rate in patients with breast cancer (5).

The linkage between CXCR4 and HER2, both of which play important roles in breast cancer progress, provides the foundation for examining the anti-tumor effects of combinational immunotherapy by targeting both CXCR4 and HER2. Therefore in this grant we have proposed to assess the effect of combination treatment with human anti-CXCR4 Mabs we have identified and anti-HER2 antibody Herceptin on tumor growth and tumor metastasis in breast xenograft models.

Body:

We originally proposed to conduct *in vitro* and *in vivo* studies to determine if the combinational use of neutralizing human CXCR4 monoclonal antibodies (Mabs) with human anti-HER2 Mab (Herceptin) could act synergistically to treat breast cancer. However, in the last year we have encountered technical difficulties with the *in vitro* assays that would be used to identify the neutralizing CXCR4 Mab from a whole set of human CXCR4 Mabs generated by our lab.

In the original proposal, two major tasks were outlined. One of these, the *in vitro* experiment, it was divided into 5 aims :

1. Produce human anti-CXCR4 antibodies from stable CHO cell lines (Month 1-2).
2. Establish HER2 and luciferase stable expressing MDA-MB-231 breast cell line (Month 1-2).

3. Perform FACS analysis to evaluate down-regulation of CXCR4 expression with a series of different antibody treatments (Months 3).
4. Perform in vitro chemotaxis and invasion assays to evaluate whether synergistic inhibitory effects of antibodies against CXCR4 and HER2 are seen on the migration and invasion activity of breast cancer cells (Months 4-5).
5. Determine if the combination treatment cells with human anti-CXCR4 and Her2 Mabs will be more potent than a single agent in inhibiting breast cancer cell proliferation in vitro (Months 6).

In the past year, we have finished aims #1 and aim #2:

Aim #1, we have produced enough antibodies against CXCR4 for in vitro assays and animal studies from CHO stable cell lines, they are Mab 33 and 48.

Aim #2, we have established Her2 and luciferase stable expressing MDA-MB-231 breast cell line by transducing the cells with Her2 and Luciferase expressing retroviral vectors. We also have established MDA-MB-231 cell line which expresses high level of Her2 and CXCR4. A luciferase expressing derivative cell line of it was also established.

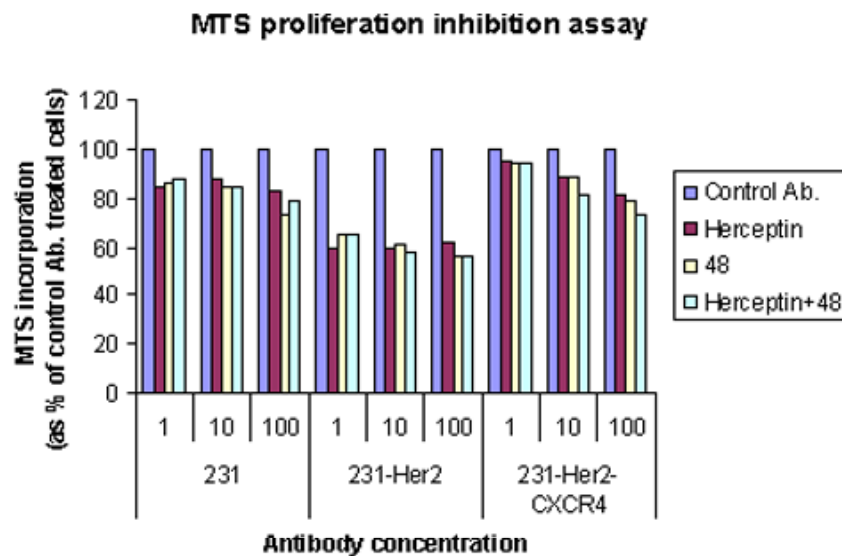
What we have finished and the difficulties we have encountered in aims #3, 4 and #5 in the past year are listed as follows:

1 - Aim #3, we could not detect CXCR4 Mabs induced down-regulation of CXCR4 expression on breast cancer cell MDA-MB-231 and a few other breast cancer cell lines. We think the main reason for this might be that the baseline expression of CXCR4 on these breast cancer cells is low or even under the detection level of FACS analysis. Therefore we established a few high CXCR4-expression breast cancer cell lines including MDA-MB-231-CXCR4 and MCF7-CXCR4 to see if these cell lines could facilitate this assay to eventually provide us a definitive answer. However, we still could not see down-regulation of CXCR4 expression by CXCR4-Mabs on these CXCR4 high expression cell lines. Based on these data, we think this might not be a suitable assay to evaluate the function of CXCR4 Mabs.

2 - Aim #4, the in vitro chemotaxis and invasion assay. For the same reason as we mentioned above the baseline level of chemotactic and invasive activities of breast cancer cell, MDA-MB-231 (and a few others we have tried), are too low to be able to provide a sensitive screening assay for determining the neutralizing activity of CXCR4 Mabs. Therefore, we expected that high-CXCR4 expression on breast cancer cells could increase the sensitivity of this assay. Our recent experimental data showed that high-CXCR4-expression MDA-MB-231 might have increased chemotactic activity to CXCR4 ligand, SDF-1 α , but we often get non-repeatable results with this assay. We will still need to figure out well-defined conditions for obtaining solid and repeatable results.

3 - Aim #5, because of the technical difficulties we have had on aims # 3 and #4. This aim has been unavoidably delayed. However, we have tried one leading CXCR4 Mab 48, which has the

highest binding activity to CXCR4, to see if combination treatment cells with it and Her2 Mab will be more potent than a single agent in inhibiting breast cancer cell proliferation. The preliminary result is shown below. The CXCR4 antibody 48 or Herceptin were shown to inhibit cell proliferation of MBA-MD-231-Her2 cells by about 40% at concentrations as low as 1 $\mu\text{g/ml}$. In contrast, significant inhibition of the Herceptin and 48 on parental MBA-MD-231 cells and MBA-MD-231-Her2-CXCR4 cells were not observed. The levels of growth inhibition mediated by Herceptin on parental cells and Her2 high expression cells are consistent with the results of another study (6). We did not found synergistic or additive proliferation inhibition effect of the combination of 48 and Herceptin antibodies in this assay. This finding still needs to be confirmed with more repeats.



The other task we have originally proposed is *in vivo* animal study to perform *in vivo* animal studies to evaluate if the combined use of human Mabs against HER2 and CXCR4 can synergistically inhibit growth of xenografts, lung metastases and prolong overall survival (Months 7-12). Because our major efforts have been focused on the *in vitro* assays in the past year it has not been started as planned. We are now planning to start the animal study in parallel with the *in vitro* studies instead of waiting until *in vitro* studies are finished. We have completed a pilot study with one CXCR4 antibody (without knowing its neutralizing activities *in vitro*) to test if it can reduce the metastasis of breast cancer cells in an animal. A promising preliminary result has been obtained from this study. We are encouraged to go ahead to test the inhibitory effects of the CXCR4 Mabs in combination with Herceptin to treat breast cancer in animals.

Key research accomplishments:

- Produced sufficient human anti-CXCR4 Mabs for the entire study.
- Established cell lines which are necessary to perform both in vitro and in vivo animal studies.
- Evaluated down-regulation of CXCR4 expression by CXCR4 Mabs on breast cancer cell lines. We found this might not be a suitable assay to evaluate the function of CXCR4 Mabs.
- We found one CXCR4 Mab has the potential of cell growth inhibition activity in vitro.
- A pilot study of one CXCR4 Mab preliminarily showed activity of inhibiting lung metastasis in a mouse model.

Reportable outcomes: A manuscript, abstract or presentation has not been resulted from this research. We have developed Herceptin, CXCR4 and Lucifase high expressing MBA-MD-231 cell lines.

Conclusions: Our anti-CXCR4 Mabs have demonstrated anti-proliferative effects on MBA-MD-231-her2-CXCR4 cells that is equal to anti-Her2 Mab although additive or synergistic inhibition could not be demonstrated. However, it is not clear if anti-proliferative activity is directly responsible for the clearing of tumor cells in vivo. Indeed, immune mediated killing by antibody dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) may be equally or more important. However, at the completion of our planned in vitro and in vivo studies in the coming year we will be able to answer these important questions and determine if anti-CXCR4/Her2 immunotherapy is superior to either immunotherapy alone.

Appendices: None

Reference:

1. Muller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME, McClanahan T, Murphy E, Yuan W, Wagner SN, Barrera JL, Mohar A, Verastegui E, Zlotnik A. Involvement of chemokine receptors in breast cancer metastasis. *Nature*. 2001, 410: 50-56.
2. Smith MC, Luker KE, Garbow JR, Prior JL, Jackson E, Piwnica-Worms D, Luker GD. CXCR4 regulates growth of both primary and metastatic breast cancer. *Cancer Res*. 2004, 64(23): 8604-8612.
3. Lapteva N, Yang AG, Sanders DE, Strube RW, Chen SY. CXCR4 knockdown by small interfering RNA abrogates breast tumor growth in vivo. *Cancer Gene Ther*. 2005,12(1): 84-89.

4. Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, Fleming T, Eiermann W, Wolter J, Pegram M, Baselga J, Norton L. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med*. 2001 Mar 15;344(11):783-92.
5. Li YM, Pan Y, Wei Y, Cheng X, Zhou BP, Tan M, Zhou X, Xia W, Hortobagyi GN, Yu D, Hung MC. Upregulation of CXCR4 is essential for HER2-mediated tumor metastasis. *Cancer Cell*. 2004;6(5): 459-469.
6. du Manoir JM, Francia G, Man S, Mossoba M, Medin JA, Vilorio-Petit A, Hicklin DJ, Emmenegger U, Kerbel RS. Strategies for delaying or treating in vivo acquired resistance to trastuzumab in human breast cancer xenografts. 2006, *Clin Cancer Res*. 12:904-16.